

**NUCLEOTIDE SEQUENCES MEDIATING MALE FERTILITY  
AND METHOD OF USING SAME**

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**BACKGROUND OF THE INVENTION**

Development of hybrid plant breeding has made possible considerable advances in quality and quantity of crops produced. Increased yield and combination of desirable characteristics, such as resistance to disease and insects, heat and drought tolerance, along with variations in plant composition are all possible because of hybridization procedures. These procedures frequently rely heavily on providing for a male parent contributing pollen to a female parent to produce the resulting hybrid.

Field crops are bred through techniques that take advantage of the plant's method of pollination. A plant is self-pollinating if pollen from one flower is transferred to the same or another flower of the same plant. A plant is cross-pollinated if the pollen comes from a flower on a different plant.

In *Brassica*, the plant is normally self sterile and can only be cross-pollinated. In self-pollinating species, such as soybeans and cotton, the male and female plants are anatomically juxtaposed. During natural pollination, the male reproductive organs of a given flower pollinate the female reproductive organs of the same flower.

Maize plants (*Zea mays L.*) present a unique situation in that they can be bred by both self-pollination and cross-pollination techniques. Maize has male flowers, located on the tassel, and female flowers, located on the ear, on the same plant. It can self or cross pollinate. Natural pollination occurs in maize when wind blows pollen from the tassels to the silks that protrude from the tops of the incipient ears.

A reliable method of controlling fertility in plants would offer the opportunity for improved plant breeding. This is especially true for development of maize hybrids, which

relies upon some sort of male sterility system and where a female sterility system would reduce production costs.

The development of maize hybrids requires the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent selection are two of the breeding methods used to develop inbred lines from populations. Breeding programs combine desirable traits from two or more inbred lines or various broad-based sources into breeding pools from which new inbred lines are developed by selfing and selection of desired phenotypes. A hybrid maize variety is the cross of two such inbred lines, each of which may have one or more desirable characteristics lacked by the other or which complement the other. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which have commercial potential. The hybrid progeny of the first generation is designated F<sub>1</sub>. In the development of hybrids only the F<sub>1</sub> hybrid plants are sought. The F<sub>1</sub> hybrid is more vigorous than its inbred parents. This hybrid vigor, or heterosis, can be manifested in many ways, including increased vegetative growth and increased yield.

Hybrid maize seed can be produced by a male sterility system incorporating manual detasseling. To produce hybrid seed, the male tassel is removed from the growing female inbred parent, which has been planted in alternating rows with the male inbred parent. Consequently, providing that there is sufficient isolation from sources of foreign maize pollen, the ears of the female inbred will be fertilized only with pollen from the male inbred. The resulting seed is therefore hybrid and will form hybrid plants.

Environmental variation in plant development can result in plants tasseling after manual detasseling of the female parent is completed. Or, a detasseler might not completely remove the tassel of a female inbred plant. In any event, the result is that the female plant will successfully shed pollen and some female plants will be self-pollinated. This will result in seed of the female inbred being harvested along with the hybrid seed, which is normally produced. Female inbred seed is not as productive as F<sub>1</sub> seed. In addition, the presence of female inbred seed can represent a germplasm security risk for the company producing the hybrid.

Alternatively, the female inbred can be mechanically detasseled by machine. Mechanical detasseling is approximately as reliable as hand detasseling, but is faster and less costly. However, most detasseling machines produce more damage to the plants than hand detasseling. Thus, no form of detasseling is presently entirely satisfactory, and a need  
5 continues to exist for alternatives, which further reduce production costs and eliminate self-pollination in the production of hybrid seed.

A reliable system of genetic male sterility would provide advantages. The laborious detasseling process can be avoided in some genotypes by using cytoplasmic male-sterile (CMS) inbreds. In the absence of a fertility restorer gene, plants of a CMS  
10 inbred are male sterile as a result of factors resulting from the cytoplasmic, as opposed to the nuclear, genome. Thus, this characteristic is inherited exclusively through the female parent in maize plants, since only the female provides cytoplasm to the fertilized seed. CMS plants are fertilized with pollen from another inbred that is not male-sterile. Pollen from the second inbred may or may not contribute genes that make the hybrid plants male-  
15 fertile. Usually seed from detasseled normal maize and CMS produced seed of the same hybrid must be blended to insure that adequate pollen loads are available for fertilization when the hybrid plants are grown and to insure diversity.

There can be other drawbacks to CMS. One is an historically observed association of a specific variant of CMS with susceptibility to certain crop diseases. This problem has  
20 discouraged widespread use of that CMS variant in producing hybrid maize and has had a negative impact on the use of CMS in maize in general.

One type of genetic sterility is disclosed in U.S. Patents 4,654,465 and 4,727,219 to Brar, et al. However, this form of genetic male sterility requires maintenance of multiple mutant genes at separate locations within the genome and requires a complex marker  
25 system to track the genes and make use of the system convenient. Patterson also described a genic system of chromosomal translocations which can be effective, but which are complicated. U.S. Patents No. 3,861,709 and 3,710,511.

Many other attempts have been made to improve on these drawbacks. For example, Fabijanski, et al., developed several methods of causing male sterility in plants (see EPO  
30 89/3010153.8 publication no. 329,308 and PCT application PCT/CA90/00037 published as

WO 90/08828). One method includes delivering into the plant a gene encoding a cytotoxic substance associated with a male tissue specific promoter. Another involves an antisense system in which a gene critical to fertility is identified and an antisense to the gene inserted in the plant. Mariani, et al. also shows several cytotoxic and antisense systems. See EP  
5 89/401, 194. Still other systems use "repressor" genes which inhibit the expression of another gene critical to male sterility. PCT/GB90/00102, published as WO 90/08829.

A still further improvement of this system is one described at U.S. Patent No. 5,478,369 (incorporated herein by reference) in which a method of imparting controllable male sterility is achieved by silencing a gene native to the plant that is critical for male  
10 fertility and replacing the native DNA with the gene critical to male fertility linked to an inducible promoter controlling expression of the gene. The plant is thus constitutively sterile, becoming fertile only when the promoter is induced and its attached male fertility gene is expressed.

As noted, an essential aspect of much of the work underway with male sterility  
15 systems is the identification of genes impacting male fertility.

Such a gene can be used in a variety of systems to control male fertility including those described herein. Previously, a male fertility gene has been identified in *Arabidopsis thaliana* and used to produce a male sterile plant. Aarts, et al., "Transposon Tagging of a Male Sterility Gene in Arabidopsis", Nature, 363:715-717 (Jun. 24, 1993). U.S. Patent No.  
20 5,478,369 discloses therein one such gene impacting male fertility. In the present invention the inventors provide novel DNA molecules and the amino acid sequence encoded that are critical to male fertility in plants. The inventors also provide a promoter of the gene and its essential sequences. These can be used in any of the systems where control of fertility is useful, including those described above.

25 Thus, one object of the invention is to provide a nucleic acid sequence, the expression of which is critical to male fertility in plants.

Another object of the invention is to provide a DNA molecule encoding an amino acid sequence, the expression of which is critical to male fertility in plants.

Yet another object of the invention is to provide a promoter of such nucleotide  
30 sequence and its essential sequences.

A further object of the invention is to provide a method of using such DNA molecules to mediate male fertility in plants.

Further objects of the invention will become apparent in the description and claims that follow.

## SUMMARY OF THE INVENTION

This invention relates to nucleic acid sequences, and, specifically, DNA molecules and the amino acid encoded by the DNA molecules, which are critical to male fertility. A promoter of the DNA is identified, as well as its essential sequences. It also relates to use of such DNA molecules to mediate fertility in plants.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. is a locus map of the male sterility gene BS92-7.

FIG. 2. is a gel of a Southern Blot analysis of *EcoRI* digested DNA from a Mu family segregating for male sterility and hybridized with a Mu1 probe.

FIG. 3. is a Northern Blot analysis gel of total RNA from various tissues hybridized with a *PstI/BglIII* fragment from the BS92-7 clone.

FIG 4 shows the nucleotide and protein sequences of the cDNA of BS92-7 (The cDNA is SEQ ID NO: 1, the protein is SEQ ID NO: 2).

FIG. 5 is the genomic BS92-7 sequence (the nucleotide sequence is also referred to as SEQ ID NO: 3 and the protein as SEQ ID NO: 4).

FIG 6 is a comparisons of the genomic BS92-7 sequence with the cDNA Part 1 is bases 301 to 450 of SEQ ID NO: 3 and bases 1 to 117 of SEQ ID NO: 1. Part 2 is bases 501 to 750 of SEQ ID NO: 3 and bases 118 to 290 of SEQ ID NO: 1. Part 3 is bases 851 to 1050 of SEQ ID NO: 3 and bases 291 to 487 of SEQ ID NO: 1. Part 4 is bases 1151 to 1350 of SEQ ID NO: 3 and bases 488 to 648 of SEQ ID NO: 1. Part 5 is bases 1401 to 1650 of SEQ ID NO: 3 and bases 649 to 841 of SEQ ID NO: 1. Part 6 is bases 1701 to 2140 of SEQ ID NO: 3 and bases 842 to 1197 of SEQ ID NO: 1.

FIG. 7. is a Northern analysis gel showing developmental gene expression in microsporogenesis of the gene BS92-7.

FIG. 8 is the full length promoter of BS92-7 (SEQ ID NO: 5)

FIG. 9. is a bar graph showing luciferase activity after substitution by restriction site linker scanning of select small (9-10bp) regions of the BS92-7 essential promoter fragment.

FIG. 10 shows an essential region of the BS92-7 promoter (SEQ ID NO: 6).

FIG 11 shows a diagram of a construct used to express the MS45 male fertility gene using the BS7 promoter, and an immunoblot analysis of the MS45 protein from maize plants transformed with the construct.

FIG 12 is a comparison of BS92-7 sorghum tassel (DNA is SEQ ID NO: 7 and protein is SEQ ID NO: 8) and BS92-7 maize cDNA (DNA is bases 29 to 731 of SEQ ID NO: 1 and protein is residues 10 to 244 of SEQ ID NO: 2). ~~(the sorghum DNA is SEQ No. 7 and the protein is SEQ ID NO. 8).~~

### DISCLOSURE OF THE INVENTION

All references referred to are incorporated herein by reference.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless mentioned otherwise, the techniques employed or contemplated therein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting.

Genetic male sterility results from a mutation, suppression, or other impact to one of the genes critical to a specific step in microsporogenesis, the term applied to the entire process of pollen formulation. These genes can be collectively referred to as male fertility genes (or, alternatively, male sterility genes). There are many steps in the overall pathway where gene function impacts fertility. This seems aptly supported by the frequency of genetic male sterility in maize. New alleles of male sterility mutants are uncovered in materials that range from elite inbreds to unadapted populations. To date, published genetic male sterility research has been mostly descriptive. Some efforts have been made to establish the mechanism of sterility in maize, but few have been satisfactory. This should not be surprising given the number of genes that have been identified as being responsible for male sterility. One mechanism is unlikely to apply to all mutations.

At Patent No. 5,478,369 there is described a method by which a male sterility gene was tagged on maize chromosome 9. Previously, the only described male sterility gene on chromosome 9 was MS2, which has never been cloned and sequenced. *See* Albertsen, M. and Phillips, R.L., “Developmental Cytology of 13 Genetic Male Sterile Loci in Maize” *Canadian Journal of Genetics & Cytology* 23:195-208 (Jan. 1981). The only fertility gene cloned before that had been the *Arabidopsis* gene described at Aarts, et al., *supra*.

The BS92-7 gene described herein is located on maize chromosome 7 and is critical to male fertility. The locus map is represented at Figure 1. It can be used in the systems described above, and other systems impacting male fertility.

The maize family cosegregating for sterility was named BS92-7 and was found to have an approximately 7.0 Kb *EcoRI* fragment that hybridized with a *MuI* probe. A genomic clone from the family was isolated which contained a *MuI* transposon. A probe made from DNA bordering the transposon was found to hybridize to the same ~7.0 Kb *EcoRI* fragment. This probe was used to isolate cDNA clones from a tassel cDNA library. The cDNA for BS92-7 is 1230 bp, and the *Mu* insertion occurred in exon 2 of the gene. Expression patterns, as determined by Northern analysis, show tassel specificity with peak expression highest at about the quartet to quartet release stages of microsporogenesis.

Further, it will be evident to one skilled in the art that variations, mutations, derivations including fragments smaller than the entire sequence set forth may be used which retain the male sterility controlling properties of the gene. One of ordinary skill in the art can readily assess the variant or fragment by its introduction into plants homozygous for a stable male sterile allele of BS92-7, followed by observation of the plant's male tissue development.

The invention also includes those nucleotide sequences which selectively hybridize to BS92.7 nucleotide sequences under stringent conditions. In referring to a sequence that “selectively hybridizes” with BS92-7, the term includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to the specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are target-sequence-dependent and will differ depending on the structure of the polynucleotide. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to a probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, probes of this type are in a range of about 1000 nucleotides in length to about 250 nucleotides in length.

An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). See also Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. Generally, stringent wash temperature conditions are selected to be about 5°C to about 2°C lower than the melting point (T<sub>m</sub>) for the specific sequence at a defined ionic strength and pH. The melting point, or denaturation, of DNA occurs over a narrow temperature range and represents the disruption of the double helix into its complementary single strands. The process is described by the temperature of the midpoint of transition, T<sub>m</sub>, which is also called the melting temperature. Formulas are available in the art for the determination of melting temperatures.

Preferred hybridization conditions for the nucleotide sequence of the invention include hybridization at 42°C in 50%(w/v) formamide, 6X SSC, 0.5%(w/v) SDS, 100(g/ml) salmon sperm DNA. Exemplary low stringency washing conditions include hybridization at 42°C in a solution of 2X SSC, 0.5% (w/v) SDS for 30 minutes and



repeating. Exemplary moderate stringency conditions include a wash in 2X SSC, 0.5% (w/v) SDS at 50°C for 30 minutes and repeating. Exemplary high stringency conditions include a wash in 2X SSC, 0.5% (w/v) SDS, at 65°C for 30 minutes and repeating. Sequences that correspond to the promoter of the present invention may be obtained using all the above conditions. For purposes of defining the invention, the high stringency conditions are used.

Promoter regions can be readily identified by one skilled in the art. The putative start codon containing the ATG motif is identified and upstream from the start codon is the presumptive promoter. By "promoter" is intended a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. A promoter can additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. It is recognized that having identified the nucleotide sequences for the promoter region disclosed herein, it is within the state of the art to isolate and identify further regulatory elements upstream of the TATA box from the particular promoter region identified herein. Thus the promoter region disclosed herein is generally further defined by comprising upstream regulatory elements such as those responsible for tissue and temporal expression of the coding sequence, enhancers and the like. In the same manner, the promoter elements which enable expression in the desired tissue such as male tissue can be identified, isolated, and used with other core promoters to confirm male tissue-preferred expression.

The isolated promoter sequence of the present invention can be modified to provide for a range of expression levels of the heterologous nucleotide sequence. Less than the entire promoter region can be utilized and the ability to drive anther-preferred expression retained. However, it is recognized that expression levels of mRNA can be decreased with deletions of portions of the promoter sequence. Thus, the promoter can be modified to be a weak or strong promoter. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts.

Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts. Generally, at least about 30 nucleotides of an isolated promoter sequence will be used to drive expression of a nucleotide sequence. It is recognized that to increase transcription levels, enhancers can be utilized in combination with the promoter regions of the invention. Enhancers are nucleotide sequences that act to increase the expression of a promoter region. Enhancers are known in the art and include the SV40 enhancer region, the 35S enhancer element, and the like.

Smaller fragments may yet contain the regulatory properties of the promoter so identified and deletion analysis is one method of identifying essential regions. Deletion analysis can occur from both the 5' and 3' ends of the regulatory region. Fragments can be obtained by site-directed mutagenesis, mutagenesis using the polymerase chain reaction and the like. (*See, Directed Mutagenesis: A Practical Approach* IRL Press (1991)). The 3' deletions can delineate the essential region and identify the 3' end so that this region may then be operably linked to a core promoter of choice. Once the essential region is identified, transcription of an exogenous gene may be controlled by the essential region plus a core promoter. The core promoter can be any one of known core promoters such as the Cauliflower Mosaic Virus 35S or 19S promoter (U.S. Patent No. 5,352,605), ubiquitin promoter (U.S. Patent No. 5,510,474) the IN2 core promoter (U.S. Patent No. 5,364,780) or a Figwort Mosaic Virus promoter (Gruber, et al. "Vectors for Plant Transformation" *Methods in Plant Molecular Biology and Biotechnology* Glick et al. eds, CRC Press pp.89-119 (1993)).

The regulatory region of BS92-7 has been identified as including the about 270 base pair region upstream of the putative TATA box. (See Figure 8.) Further, using the procedures outlined above, it has been determined that an essential region of the promoter includes the -112 to -93 bp upstream of the TATA box.

Promoter sequences from other plants may be isolated according to well-known techniques based on their sequence homology to the promoter sequence set forth herein. In these techniques, all or part of the known promoter sequence is used as a probe which selectively hybridizes to other sequences present in a population of cloned genomic DNA

fragments (i.e. genomic libraries) from a chosen organism. Methods are readily available in the art for the hybridization of nucleic acid sequences.

The entire promoter sequence or portions thereof can be used as a probe capable of specifically hybridizing to corresponding promoter sequences. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes can be used to amplify corresponding promoter sequences from a chosen organism by the well-known process of polymerase chain reaction (PCR). This technique can be used to isolate additional promoter sequences from a desired organism or as a diagnostic assay to determine the presence of the promoter sequence in an organism. Examples include hybridization screening of plated DNA libraries (either plaques or colonies; see e.g. Innis et al., eds., (1990) PCR Protocols, A Guide to Methods and Applications, Academic Press).

Further, the promoter of the present invention can be linked with nucleotide sequences other than the BS92-7 gene to express other heterologous nucleotide sequences. The nucleotide sequence for the promoter of the invention, as well as fragments and variants thereof, can be provided in expression cassettes along with heterologous nucleotide sequences for expression in the plant of interest, more particularly in the male tissue of the plant. Such an expression cassette is provided with a plurality of restriction sites for insertion of the nucleotide sequence to be under the transcriptional regulation of the promoter. These expression cassettes are useful in the genetic manipulation of any plant to achieve a desired phenotypic response. Examples of other nucleotide sequences which can be used with the BS92-7 promoter as the exogenous gene of the expression vector include complementary nucleotidic units such as antisense molecules (callase antisense RNA, barnase antisense RNA and chalcone synthase antisense RNA, Ms45 antisense RNA), ribozymes and external guide sequences, an aptamer or single stranded nucleotide sequences. The exogenous nucleotide sequence can also encode auxins, rol B, cytotoxins, diphtheria toxin, DAM methylase, avidin, or may be selected from a prokaryotic regulatory system. By way of example, Mariani, et al., Nature; Vol. 347; pp. 737; (1990), have shown that expression in the tapetum of either *Aspergillus oryzae* RNase-T1 or an

RNase of *Bacillus amyloliquefaciens*, designated "barnase," induced destruction of the tapetal cells, resulting in male infertility. Quaas, et al., Eur. J. Biochem. Vol. 173: pp. 617 (1988), describe the chemical synthesis of the RNase-T1, while the nucleotide sequence of the barnase gene is disclosed in Hartley, J. Molec. Biol.; Vol. 202: pp. 913 (1988). The *rolB* gene of *Agrobacterium rhizogenes* codes for an enzyme that interferes with auxin metabolism by catalyzing the release of free indoles from indoxyl- $\beta$ -glucosides. Estruch, et al., EMBO J. Vol. 11: pp. 3125 (1991) and Spena, et al., Theor. Appl. Genet.; Vol. 84: pp. 520 (1992), have shown that the anther-specific expression of the *rolB* gene in tobacco resulted in plants having shriveled anthers in which pollen production was severely decreased and demonstrated the *rolB* gene is another gene that is useful for the control of pollen production. Slightom, et al., J. Biol. Chem. Vol. 261: pp. 108 (1985), disclose the nucleotide sequence of the *rolB* gene. DNA molecules encoding the diphtheria toxin gene can be obtained from the American Type Culture Collection (Rockville, MD), ATCC No. 39359 or ATCC No. 67011 and see Fabijanski, et al., E.P. Appl. No. 90902754.2, "Molecular Methods of Hybrid Seed Production" for examples and methods of use. The DAM methylase gene is used to cause sterility in the methods discussed at U.S. Patent Nos. 5,792,853, 5,689,049 and PCT/US95/15229 Cigan, A.M. and Albertsen, M.C., "Reversible Nuclear Genetic System for Male Sterility in Transgenic Plants". Also see discussion of use of the avidin gene to cause sterility at U.S. Patent No. 5,962,769.

The invention includes vectors with the BS92-7 gene. A vector is prepared comprising the BS92-7 gene, a promoter that will drive expression of the gene in the plant and a terminator region. As noted, the promoter in the construct may be the native promoter or a substituted promoter which will provide expression in the plant. The choice of promoter will depend upon the use intended of the gene. The promoter in the construct may be an inducible promoter, so that expression of the sense or antisense molecule in the construct can be controlled by exposure to the inducer.

Other components of the vector may be included, also depending upon intended use of the gene. Examples include selectable markers, targeting or regulatory sequences, stabilizing or leader sequences, etc.. General descriptions and examples of plant expression vectors and reporter genes can be found in Gruber, et al., "Vectors for Plant

Transformation” in Method in Plant Molecular Biology and Biotechnology, Glick et al eds; CRC Press pp. 89-119 (1993). The selection of an appropriate expression vector will depend upon the host and the method of introducing the expression vector into the host. The expression cassette can also include at the 3’ terminus of the heterologous nucleotide sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region can be native with the promoter nucleotide sequence of the present invention, can be native with the DNA sequence of interest, or can be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. *See also*, Guerineau et al. Mol. Gen. Genet. 262:141-144 (1991); Proudfoot Cell 64:671-674 (1991); Sanfacon et al. Genes Dev. 5:141-149 (1991); Mogen et al. Plant Cell 2:1261-1272 (1990); Munroe et al. Gene 91:151-158 (1990); Ballas et al. Nucleic Acids Res. 17:7891-7903 (1989); Joshi et al. Nucleic Acid Res. 15:9627-9639 (1987).

The expression cassettes can additionally contain 5’ leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5’ noncoding region), Elroy-Stein et al. Proc. Nat. Acad. Sci. USA 86:6126-6130 (1989); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus), Allison et al.; MDMV leader (Maize Dwarf Mosaic Virus), Virology 154:9-20 (1986); human immunoglobulin heavy-chain binding protein (BiP), Macejak et al. Nature 353:90-94 (1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), Jobling et al. Nature 325:622-625 (1987); Tobacco mosaic virus leader (TMV), Gallie et al. (1989) Molecular Biology of RNA, pages 237-256; and maize chlorotic mottle virus leader (MCMV) Lommel et al. Virology 81:382-385 (1991). *See also* Della-Cioppa et al. Plant Physiology 84:965-968 (1987). The cassette can also contain sequences that enhance translation and/or mRNA stability such as introns.

In those instances where it is desirable to have the expressed product of the heterologous nucleotide sequence directed to a particular organelle, particularly the plastid, amyloplast, or to the endoplasmic reticulum, or secreted at the cell's surface or extracellularly, the expression cassette can further comprise a coding sequence for a transit

peptide. Such transit peptides are well known in the art and include, but are not limited to, the transit peptide for the acyl carrier protein, the small subunit of RUBISCO, plant EPSP synthase, and the like. One skilled in the art will readily appreciate the many options available in expressing a product to a particular organelle. For example, the barley alpha amylase sequence is often used to direct expression to the endoplasmic reticulum (Rogers, J. Biol. Chem. 260:3731-3738 (1985)). Use of transit peptides is well known (*e.g.*, see U.S. Patents Nos. 5,717,084; 5,728,925).

In preparing the expression cassette, the various DNA fragments can be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers can be employed to join the DNA fragments or other manipulations can be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction digests, annealing, and resubstitutions, such as transitions and transversions, can be involved.

As noted herein, the present invention provides vectors capable of expressing genes of interest under the control of the promoter. In general, the vectors should be functional in plant cells. At times, it may be preferable to have vectors that are functional in *E. coli* (*e.g.*, production of protein for raising antibodies, DNA sequence analysis, construction of inserts, obtaining quantities of nucleic acids). Vectors and procedures for cloning and expression in *E. coli* are discussed in Sambrook et al. (*supra*).

The transformation vector comprising the promoter sequence of the present invention operably linked to a heterologous nucleotide sequence in an expression cassette can also contain at least one additional nucleotide sequence for a gene to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another transformation vector.

Reporter genes can be included in the transformation vectors. Examples of suitable reporter genes known in the art can be found in, for example, Jefferson et al. (1991) in Plant Molecular Biology Manual, ed. Gelvin et al. (Kluwer Academic Publishers), pp. 1-33; DeWet et al. (1987) Mol. Cell. Biol. 7:725-737; Goff et al. (1990) EMBO J. 9:2517-

2522; Kain et al. (1995) BioTechniques 19:650-655; and Chiu et al. (1996) Current Biology 6:325-330.

Selectable marker genes for selection of transformed cells or tissues can be included in the transformation vectors. These can include genes that confer antibiotic  
 5 resistance or resistance to herbicides. Examples of suitable selectable marker genes include, but are not limited to, genes encoding resistance to chloramphenicol, Herrera Estrella et al. (1983) EMBO J. 2:987-992; methotrexate, Herrera Estrella et al. (1983) Nature 303:209-213; Meijer et al. (1991) Plant Mol. Biol. 16:807-820; hygromycin, Waldron et al. (1985) Plant Mol. Biol. 5:103-108; Zhijian et al. (1995) Plant Science  
 10 108:219-227; streptomycin, Jones et al. (1987) Mol. Gen. Genet. 210:86-91; spectinomycin, Bretagne-Sagnard et al. (1996) Transgenic Res. 5:131-137; bleomycin, Hille et al. (1990) Plant Mol. Biol. 7:171-176; sulfonamide, Guerineau et al. (1990) Plant Mol. Biol. 15:127-136; bromoxynil, Stalker et al. (1988) Science 242:419-423; glyphosate, Shaw et al. (1986) Science 233:478-481; phosphinothricin, DeBlock et al. (1987) EMBO J.  
 15 6:2513-2518.

The method of transformation/transfection is not critical to the instant invention; various methods of transformation or transfection are currently available. As newer methods are available to transform crops or other host cells they may be directly applied. Accordingly, a wide variety of methods have been developed to insert a DNA sequence  
 20 into the genome of a host cell to obtain the transcription or transcript and translation of the sequence to effect phenotypic changes in the organism. Thus, any method which provides for efficient transformation/transfection may be employed.

Methods for introducing expression vectors into plant tissue available to one skilled in the art are varied and will depend on the plant selected. Procedures for transforming a  
 25 wide variety of plant species are well known and described throughout the literature. See, for example, Miki et al, "Procedures for Introducing Foreign DNA into Plants" in Methods in Plant Molecular Biotechnology, *supra*; Klein et al, (1992) Bio/Technology 10:268; and Weising et al., (1988) Ann. Rev. Genet. 22: 421-477. For example, the DNA construct may be introduced into the genomic DNA of the plant cell using techniques such as  
 30 microprojectile-mediated delivery, Klein et al., (1987) Nature 327: 70-73; electroporation,

Fromm et al., (1985) Proc. Natl. Acad. Sci. 82: 5824; polyethylene glycol (PEG) precipitation, Paszkowski et al., (1984) EMBO J. 3: 2717-2722; direct gene transfer WO 85/01856 and EP No. 0 275 069; in vitro protoplast transformation U.S. Patent No. 4,684,611; and microinjection of plant cell protoplasts or embryogenic callus. Crossway, (1985) Mol. Gen. Genetics 202:179-185. Co-cultivation of plant tissue with *Agrobacterium tumefaciens* is another option, where the DNA constructs are placed into a binary vector system. See e.g., U.S. Patent No. 5,591,616; Ishida et al., (1996) Nature Biotechnology 14:745-750. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct into the plant cell DNA when the cell is infected by the bacteria. See, for example Horsch et al., (1984) Science 233: 496-498, and Fraley et al., (1983) Proc. Natl. Acad. Sci. 80: 4803.

Standard methods for transformation of canola are described at Moloney et al. "High Efficiency Transformation of *Brassica napus* using *Agrobacterium* Vectors" Plant Cell Reports 8:238-242 (1989). Corn transformation is described by Fromm et al, Bio/Technology 8:833 (1990) and Gordon-Kamm et al, *supra*. *Agrobacterium* is primarily used in dicots, but certain monocots such as maize can be transformed by *Agrobacterium*. See *supra* and U.S. Patent No. 5,550,318. Rice transformation is described by Hiei et al., (1992) The Plant Journal 6(2): 271-282 (1994, Christou et al, *Trends in Biotechnology* 10:239 and Lee et al, Proc. Nat'l Acad. Sci. USA 88:6389 (1991). Wheat can be transformed by techniques similar to those used for transforming corn or rice. Sorghum transformation is described at Casas et al, *supra* and sorghum by Wan et al, (1994) Plant Physicol. 104:37. Soybean transformation is described in a number of publications, including U.S. Patent no. 5,015,580.

Further detailed description is provided below by way of instruction and illustration and is not intended to limit the scope of the invention.

### Example 1

#### Identification and Cosegregation of BS92-7

Families of plants from a mutator (*Mu*) population were identified that segregated for a male sterile phenotype, with none or only a few extruded abnormal anthers, none of which had pollen present. Male sterility is expected to result from those instances where a



*Mu* element has randomly integrated into a gene responsible for some step in microsporogenesis, disrupting its expression. Plants from a segregating F<sub>2</sub> family, designated BS92-7, were grown and classified for male fertility/sterility based on the above criteria. Leaf samples were taken and subsequent DNA isolated on approximately 20 plants per phenotypic classification

Southern analysis was performed to confirm association of *Mu* with sterility. Southern analysis is a well known technique to those skilled in the art. This common procedure involves isolating the plant DNA, cutting with restriction endonucleases, fractionating the cut DNA by molecular weight on an agarose gel, and transferring to nylon membranes to fix the separated DNA. These membranes were subsequently hybridized with the *Mu*-probe fragment that was radioactively labeled with  $\alpha^{32}\text{P}$ -dCTP, and washed in an SDS solution. Southern, E., (1975) "Detection of Specific Sequences Among DNA Fragments by Gel Electrophoresis," *J. Mol. Biol.* 98:503-317. Plants from a segregating F<sub>2</sub> BS92-7 family were grown and classified for male fertility/sterility. Leaf sampling and subsequent DNA isolation was accomplished on approximately 20 plants per phenotypic classification. DNA (~7ug) from 5 fertile and 12 sterile plants was digested with *EcoRI* and subjected to electrophoresis through a 0.75% agarose gel. The digested DNA was transferred to nylon membrane via Southern transfer. The membrane was hybridized with an internal fragment from the *Mu1* transposon. Autoradiography of the membrane revealed cosegregation of a 7 Kb *EcoRI* fragment with the sterility phenotype as shown at Fig. 2. This *EcoRI* band segregated in the fertile plants suggesting a segregating homozygous-heterozygous wild type condition for the allele.

### Example 2

#### Library Construction and Screening

The process of cDNA library screenings is commonly known among those skilled in the art and is described at Sambrook, J., Fritsch, E.F., Maniatis T., et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Lab Press, Plainview, NY. Libraries were created as follows.

DNA from a sterile plant was digested with *EcoRI* and run on a preparative gel. DNA with a molecular weight between 6.0 and 8.0 kb was excised from the gel,

electroeluted and ethanol precipitated. This DNA was ligated into the Lambda Zap vector (Stratagene <sup>TM</sup>) using the manufacturer's protocol. The ligated DNA was packaged into phage particles using Gigapack Gold (Stratagene <sup>TM</sup>). Approximately 500,000 PFU were plated and lifted onto nitrocellulose membranes. Membranes were hybridized with the *Mul* probe. A pure clone was obtained after three rounds of screening. The insert was excised from the phage as a plasmid and designated BS927-8.1. A border fragment from this clone was isolated and used to reprobe the original *EcoRI* cosegregation blot. The 7.0 kb *EcoRI* fragment is homozygous in all the sterile plants, which confirms that the correct *Mu* fragment was isolated. Eight of the fertile plants are heterozygous for the 7.0 kb *EcoRI* band and a 6.2 Kb *EcoRI* band. Two of the fertile plants are homozygous for the 6.2 kb *EcoRI* band, presumably the wild type allele.

### Example 3

#### Expression Analysis and cDNA Isolation

Northern analysis can be used to detect expression of genes at various stages of microsporogenesis. Northern analysis is a commonly used technique known to those skilled in the art and is similar to Southern analysis except that mRNA instead of DNA is isolated and placed on the gel. The mRNA is then hybridized with the labeled probe. Potter, E., et al., (1981) Proc. Nat. Acad. Sci. USA 78:6662-6666, Lechelt, et al. (1989) Mol.Gen.Genet. 219:225-234. A *PstI/BglIII* fragment from BS927-8.1 was used to probe a northern blot containing kernel, immature ear, seedling and tassel RNA. A signal was seen only in tassel RNA at approximately the quartet stage of microsporogenesis as reflected at Figure 3. The transcript is about 1.4 kb in length. The same probe was also used to screen a cDNA library constructed from mRNA isolated from meiotic to late uninucleate staged anthers. Two clones, designated BS927-4.1 and BS927-9.1, were isolated from the library.

### Example 4

#### Sequence Analysis

BS927-8.1 genomic clone and the two cDNA clones, BS927-4.1 and BS927-9.1, were sequenced. Sequences methods are well known in the art and this sequencing was accomplished by Loftstrand Labs Limited using the methods discussed at Sanger, F., Nicklen, S., Coulson, A.R. (1977) "DNA Sequences with chain terminating inhibitors"

Proc. Natl. Acad. Sci. USA 74:5463-5467. The two cDNA clones differ at the 5' end of the molecule. BS927-4.1 contains a TCTC repeat, whereas the BS927-9.1 does not. When these sequences are compared to the genomic clone, the TCTC repeat is not present and probably represents a cloning artifact in the BS927-4.1 cDNA. The cDNA/genomic comparison reveals six exons and five introns are present in the genomic clone. The cDNA sequence is set forth in Figure 4 and the genomic shown in Figure 5. A comparison of the genomic and cDNA is provided in Figure 6 and demonstrated a 95.95% identity. The *Mul* insertion occurs in exon2. There is a putative Met start codon at position 320 in the genomic clone. Since both cDNAs lack this Met codon, they did not represent full length genes. Subsequent cDNA screening with BS927-4.1 allowed for the isolation of clone BS927-11.1. This clone was only sequenced at the 5' end to determine its start point. It was determined that BS927-11.1 lacks 2 bases of the Met codon (ATG) and is the longest cDNA isolated. Further expression studies were done using the BS927-4.1 cDNA probe against a Northern containing mRNA at discrete stages of microsporogenesis. Signal is detected from meiosis II/quartet to mid-uninucleate, with maximal signal being at quartet to quartet release as shown at Figure 7.

### Example 5

#### Identification of Promoter and its Essential Regions

Comparison of the BS927-8.1 genomic clone with the cDNA clones BS927-11.1, BS927-4.1 and BS927-9.1 allowed identification of introns and exons in BS927. This in turn permitted identification of ORFs, one of which extended through most of the cDNA sequence, and was most likely the protein coding sequence of the BS927 gene. Testing for codon preference and non-randomness in the third position of each codon confirmed that this was the likely protein-coding ORF. At the amino acid level, the protein that would be encoded has 52% similarity (42% identity) with the maize gene A1, which encodes dihydroflavanol reductase and is required for synthesis of anthocyanins and phlobaphenes.

Regulatory regions of anther genes, such as promoters, may be identified in genomic subclones using functional analysis, usually verified by the observation of reporter gene expression in anther tissue and a lower level or absence of reporter gene expression in non-anther tissue. The possibility of the regulatory regions residing "upstream" or 5' ward

of the transcriptional start site can be tested by subcloning a DNA fragment that contains the upstream region into expression vectors for transient expression experiments. It is expected that smaller subgenomic fragments may contain the regions essential for male-tissue preferred expression. For example, the essential regions of the CaMV 19S and 35S promoters have been identified in relatively small fragments derived from larger genomic pieces as described in U.S. Pat. No. 5,352,605.

The selection of an appropriate expression vector with which to test for functional expression will depend upon the host and the method of introducing the expression vector into the host and such methods are well known to one skilled in the art. For eukaryotes, the regions in the vector include regions that control initiation of transcription and control processing. These regions are operably linked to a reporter gene such as *UidA*, encoding  $\beta$ -glucuronidase (GUS), or luciferase. General descriptions and examples of plant expression vectors and reporter genes can be found in Gruber, et al., *supra*. GUS expression vectors and GUS gene cassettes are commercially available from Clontech, Palo Alto, CA, while luciferase expression vectors and luciferase gene cassettes are available from Promega Corporation, Madison, WI. Ti plasmids and other *Agrobacterium* vectors are described in Ishida, Y., et al., (1996) Nature Biotechnology; Vol. 14; pp. 745-750; and in U.S. Pat. No. 5,591,616.

Expression vectors containing putative regulatory regions located in genomic fragments can be introduced into intact tissues such as staged anthers, embryos or into callus. Methods of DNA delivery include microprojectile bombardment, DNA injection, electroporation and *Agrobacterium*-mediated gene transfer (*see* Gruber, et al., *supra*, U.S. Pat. No. 5,591,616, and Ishida, Y., et al., *supra*). General methods of culturing plant tissues are found in Gruber, et al., *supra*.

For the transient assay system, staged, isolated anthers are immediately placed onto tassel culture medium (Pareddy, D.R. and J.F. Petelino, (1989) Crop Sci. J.; Vol. 29; pp. 1564-1566;) solidified with 0.5% Phytigel (Sigma, St. Louis) or other solidifying media. The expression vector DNA is introduced within 5 hours preferably by microprojectile-mediated delivery with 1.2  $\mu$ m particles at 1000 -1100 Psi. After DNA delivery, the anthers are incubated at 26°C upon the same tassel culture medium for 17 hours and

analyzed by preparing a whole tissue homogenate and assaying for GUS or for luciferase activity (see Gruber, et al., *supra*).

Upstream of the likely translational start codon of BS927, only 319 bp of BS927 DNA were present in the genomic clone BS927-8.1. Translational fusions via an engineered *NcoI* site were generated with reporter genes encoding luciferase and  $\beta$ -glucuronidase to test whether this fragment of DNA had promoter activity in transient expression assays of bombarded plant tissues. Activity was demonstrated in anthers and not in coleoptiles, roots and calli, suggesting anther-preferred or anther-specific promoter activity.

A reasonable TATA box was observed by inspection upstream of the translational start codon. The genomic clone BS927-8.1 thus includes only about 266 bp upstream of the putative TATA box. For typical plant genes, the start of transcription is 26-36 bp downstream of the TATA box, which would give the BS927 mRNA a 5'-nontranslated leader of only about 17-27 nucleotides (nt). The total BS927 subgenomic fragment of only 319 bp, including nontranslated leader, start of transcription, TATA box and sequences upstream of the TATA box, was thus shown to be sufficient for promoter activity. See Figure 8, which is SEQ. ID NO.5. It will be appreciated by those skilled in the art that promoter fusions with genes, open reading frames, RNA-encoding sequences and the like may be either at or close to the native start of transcription or at the start of translation or downstream of the start codon. The putative TATA box (TTTATAA) is underlined. Thus, the present invention encompasses a DNA molecule having a nucleotide sequence of SEQ ID NO. 5 (or those with sequence identity or which hybridize thereto) and having the function of a male tissue-preferred regulatory region.

Deletion analysis can occur from both the 5' and 3' ends of the regulatory region: fragments can be obtained by site-directed mutagenesis, mutagenesis using the polymerase chain reaction, and the like (Directed Mutagenesis: A Practical Approach; IRL Press; (1991)). The 3' end of the male tissue-preferred regulatory region can be delineated by proximity to the putative TATA box or by 3' deletions if necessary. The essential region may then be operably linked to a core promoter of choice via cloning restriction sites introduced into the region immediately upstream of the TATA box or as defined by 3' deletion analysis.

Once the essential region is identified, transcription of an exogenous gene may be controlled by the male tissue-preferred region of BS92-7 plus a core promoter. The core promoter can be any one of known core promoters such as a Cauliflower Mosaic Virus 35S or 19S promoter (U.S. Pat. No. 5,352,605), ubiquitin (U.S. Pat. No. 5,510,474), the IN2 core promoter (U.S. Pat. No. 5,364,780), or a Figwort Mosaic Virus promoter (Gruber, et al., *supra*). Preferably, the promoter is the core promoter of a male tissue-preferred gene or the CaMV 35S core promoter. More preferably, the promoter is from a male tissue-preferred gene and in particular, the BS92-7 core promoter.

Further mutational analysis, for example by linker scanning, a method well-known to the art, can identify small segments containing sequences required for anther-preferred expression. These mutations may introduce modifications of functionality such as in the levels of expression, in the timing of expression or in the tissue of expression. Mutations may also be silent and have no observable effect.

The foregoing procedures were used to identify essential regions of the BS92-7 promoter. After linking the promoter with the luciferase marker gene, mutational analyses were performed. The 319 bp promoter/nontranslated leader region of the genomic clone BS7 comprises 266bp upstream of the putative TATA box. Deletion of the upstream-most one half (approximately) of this upstream region to an *MluI* site reduced transient promoter activity about tenfold in anthers, although activity was not eliminated. Introduction of a *BglIII* site at -15 to -10 relative to the putative TATA box did not greatly affect activity, but the combination of this cloning site modification with the deletion eliminated activity.

Linker scanning was initiated from -261 through -16 relative to the putative TATA box, mostly in 10bp increments, as represented in Figure 9. The bar graph shows each 7-10 bp substituted segment on the x-axis. The y-axis shows the normalized luciferase activity as a percent of wild type promoter activity. The linker scanning constructs and wild type control included the *BglIII* cloning site. The minimal promoter is further represented in Figure 10. The TATA box is identified by underlining. A single critical region from -112 through -93 relative to the putative TATA box was observed, with additional regions having a significant impact located at -161 to -152; -141 to -132; and -92 to -83 relative to the putative TATA box. The region from about -102 to -93 relative to the putative TATA

box includes two overlapping copies of a sequence, the P motif, implicated in the function of other anther promoter upstream regions as well as promoters activated by the maize *P* gene product. The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. (Grotewald, E.,  
 5 B.J. Drummond, B. Bowen, and T. Peterson (1994) Cell 76:543-553.)

### Example 6

#### BS92-7 Promoter used to drive MS45 Gene

The MS45 gene is a male fertility gene in maize and mutations in the gene result in breakdown of microsporogenesis during vacuolation of the microspores rendering the  
 10 plants male sterile. When the cloned maize MS45 gene is introduced into such mutated male sterile plants, the gene can complement the mutation and confer male fertility. For a complete discussion of the MS45 gene and its promoter, see U.S. Patent Nos. 5,478,369 and 6,037,523, incorporated herein by reference. PHP6641 is a pUC8 plasmid containing the MS45 gene promoter and coding region including introns, nucleotides 1-3335 cloned as *NcoI* DNA fragment  
 15 upstream of the 35S::PAT selectable marker gene as described above. Site directed mutagenesis (as per Su T.S. and El-Gewely M.R. *supra*, was used to introduce a *NcoI* restriction enzyme recognition site at the translation start codon of the MS45 gene (nucleotide 1389). A 4.7 kp *HindIII-EcoRI* DNA fragment containing the mutagenized version of MS45-35S::PAT was cloned in plasmid pSB11 (pSB31 from Ishida et al *supra*.) lacking the *EcoRI* DNA fragment insert carrying the 35S GUS and 35SBAR genes resulting in  
 20 PHP10890. To produce PHP12025, the BS92.7 promoter replaced the MS45 promoter. This was then introduced into a construct, shown in Figure 11. This construct was introduced by *Agrobacterium*-mediated transformation as described above into a mutant maize plant which was male sterile as a result of a mutation of the MS45 gene. All 22 events generated from the transformation were restored to a male-fertile phenotype. Immunoblot analysis of  
 25 the MS45 protein from the plants is also shown in Figure 11. MS45 protein from an inbred expressing wild-type MS45 gene served as the control. Protein extracts were isolated from anthers staged at tetrad release to early vacuolate stages of microspore development. The MS45 protein was expressed to nearly wild-type levels when transcribed from the maize BS92.7 promoter. Thus the BS92.7 promoter was able to drive appropriate expression of a  
 30 gene required to restore fertility to a male-sterile mutant line. Table 1 summarizes the results below.

Table 1

Construct	Description	%Male-Fertile	%Male-Sterile	Number of events
PHP10890	MS45::MS45	100%		11
PHP12205	BS92.7::MS45	100%		22

Example 75                    Impacting male fertility using the BS7 promoter

In addition, the BS92.7 promoter was able to drive expression of a cytotoxic gene to confer male sterility on a male-fertile genotype. The constructs to produce male sterile plants were developed as follows. The *E. coli* DNA adenine methylase gene (DAM gene) as described at U.S. Patent No. 5,792,853, was used. MS45:DAM-35SPAT(PHP12634)

The DAM gene was modified by site-directed mutagenesis (Su and El-Gewley, (1988) Gene 69:81-89) and a *SmaI* site introduced at nucleotide 186, nine nucleotides 5' to the initiating codon ATG of the DAM gene. The *NcoI* site of a 1.4 kb *HindIII-NcoI* fragment containing the maize MS45 promoter found on plasmid PHP6054 was filled-in with dNTPs using T4 DNA polymerase and ligated to the *SmaI* site contained at the 5' end of the DAM gene. Transcription of this gene was terminated by the addition of the 3' sequences from the potato proteinase inhibitor II gene (PinII) (nucleotides 2-310; An et al., (1989) Plant Cell 1:115-122).

To construct the maize transformation vector PHP12634 (MS45::DAM-35SPAT), the 2.5 kb chimeric gene containing the MS45 promoter, the DAM gene and PinII 3' non-translated region was cloned as a *HindIII-NcoI* fragment upstream of the 35S::PAT gene in the vector pSB11 (pSB31 from Ishida et al., *supra*, lacking the *EcoRI* fragment insert carrying the 35SGUS and 35SBAR genes). The PAT gene encodes the enzyme phosphinothricin acetyl-transferase (PAT) from *Streptomyces viridochromagenes* (nucleotides 6 to 557). See, EP 0 275 957 A; Genbank accession number A02774. It was placed under the transcriptional regulation of the cauliflower mosaic virus (CAMV) 35S promoter and terminator regions (nucleotides 6906



to 7439, and 7439-7632). See U.S. Patent No. 5,352,605 and Franck et al., (1980) Cell 21:285-294. The 35S::PAT component was contained on an *NcoI-KpnI* fragment as described in the expression cassette pDH51 (Pietrzak et al., (1986) Nucleic Acids Res. 14:5857-5868).

- 5            PHP12635 (BS7:DAM/35S:PAT) was constructed by replacing the MS45 promoter in PHP12634 with the BS7 promoter .

             In the following experiment the BS92.7 promoter was used to direct the transcription of the sterility gene, Dam-methylase (DAM) . A construct using the MS45 promoter for transcription of DAM was also transformed into wild-type maize. Table 2  
10 below reflects that 100% of the events containing BS92.7::DAM were male sterile, compared to the MS45::DAM construct in which only 25% of the events were male sterile.

Table 2

Construct	Description	% Male-Fertile	% Male-Sterile	Number of events
PHP12634	MS45::DAM	75%	25%	24
PHP12635	BS92.7::DAM	0	100%	10

15

### Example 8

#### Allelism

             The BS927-8.1 clone was mapped in an ECB RFLP population using *EcoRI* as the enzyme. The clone maps on the long arm of chromosome 7 between the molecular markers bnl15.40 and umc110a. The male sterile mutant, ms7, is the only known male  
20 sterile that maps to chromosome 7. Allelism crosses were initiated with the BS92-7 mutant and the ms7 stock. Progeny from this cross segregated for male sterility indicating that the same gene is responsible for the mutant phenotypes in both the BS92-7 and ms7 families.

Example 9

ms7 Isolation:

Clone ms7-5.1 was purified and sequenced using internal primers already constructed for the BS92-7 sequencing work. There is one extra Ser residue in the ms7 deduced protein as compared to the BS92-7. The major difference between the two is a 33 bp deletion in the promoter region of ms7. Transient assays of the ms7 promoter fragment show that it is active in anthers.

As noted above, the BS92-7 nucleotide sequence is allelic to the known maize male sterile mutation ms7. When BS92-7 is mutated, male sterility will result. It may be introduced into plants to impact male sterility.

Example 10

BS92-7 Sorghum Tassel RT-PCR and BS92-7 Maize cDNA Comparison

A homologue of BS92-7 was identified in sorghum. The sorghum-BS92-7 cDNA was isolated by using the maize BS92-7 gene primers in a polymerase chain reaction with sorghum flower cDNA as the template. The resultant cDNA fragment was sequenced by methods described *supra* and then compared to the BS92-7 cDNA from maize. Nucleotide sequence comparisons are set forth in Figure 12, which shows 89.1% identity between the nucleotide sequences and 94% identity between the predicted protein sequences.

As is evident from the above, the BS92-7 gene is critical to male fertility in plants. Thus it can be seen that the invention achieves at least all of its objectives.

What is claimed is:

1. An isolated nucleotide sequence comprising the BS92-7 gene.
2. An isolated nucleotide sequence that mediates male fertility in plants comprising a nucleotide sequence encoding any of the amino acid sequences of SEQ ID Nos.2, 4 or 8 and those sequences which hybridize to the nucleotide sequences encoding any of the amino acid sequences of SEQ ID Nos. 2 , 4 or 8 under highly stringent conditions.
3. An isolated DNA molecule that mediates fertility in plants comprising a nucleotide sequence of any of SEQ ID Nos.1, 3, or 7 and those sequences which hybridize to the nucleotide sequences of SEQ ID Nos. 1, 3, or 7 under highly stringent conditions.
4. A plant cell transformed by the nucleotide sequence of Claim 1.
5. A plant cell transformed by the nucleotide sequence of Claim 2.
6. A plant cell transformed by the nucleotide sequence of Claim 3.
7. A plant transformed by a nucleotide sequence of Claim 1.
8. A plant transformed by a nucleotide sequence of Claim 2.
9. A plant transformed by a nucleotide sequence of Claim 3.
10. The plant cell of Claim 4 wherein the cell is a maize cell.
11. The plant of Claim 7 wherein the plant is maize.
12. A method of impacting fertility of a plant comprising impacting the BS92-7 gene.
13. A method of impacting fertility of a plant comprising impacting a nucleotide sequence in the plant encoding the amino acid sequence of any of SEQ ID Nos 2, 4 or 8, the nucleotide sequences of any of SEQ. ID Nos. 1, 3, or 7 and those sequences which hybridize to any of said sequences under highly stringent conditions.
14. The method of Claim 12 wherein the sequence expression is repressed.
15. The method of Claim 12 wherein expression of the nucleotide sequence is repressed by mutation of the nucleotide sequence.
16. The method of Claim 12 further comprising delivering into the plant a second nucleotide sequence which represses expression of the nucleotide sequence.

17. The method of Claim 16 further comprising delivering into the plant a second nucleotide sequence molecule oriented in the antisense direction relative to the DNA molecule thereby repressing expression of the DNA molecule.
18. The method of Claim 12 wherein a native BS92-7 gene in a plant is silenced and a second BS92-7 gene linked to an inducible promoter is introduced into the plant such that the plant is constitutively male sterile and fertility is induced by inducing the promoter.
19. A method of producing hybrid seed, comprising: (a) planting in cross-pollinating juxtaposition, a first seed from a selected male fertile parent line and a second seed selected from a female parent line having male sterility produced according to the method of Claim 12; (b) growing the seed to mature plants under conditions which do not induce expression of the second DNA molecule; (c) cross-pollinating the male sterile female plant with pollen from the male fertile plant; and (d) harvesting seed from the male sterile female plant.
20. The method of claim 19 further comprising cross-fertilizing the male sterile plant with a second plant, the second plant comprising a second exogenous gene, the product of the second gene preventing disruption of the male tissue by the first exogenous gene, producing a male fertile hybrid plant.
21. The method of claim 19 wherein the gene impacting male fertility is dominant and further comprising growing the hybrid seed to produce a third male sterile parent plant; producing a fourth parent plant comprising one or more genes controlling a desired gene trait and cross-fertilizing the third and fourth parent plants to produce second hybrid seed.
22. A male fertility mediated plant produced according to the method of Claim 12.
23. A method of providing heritable externally controllable male sterility in a plant comprising linking BS92-7 in an expression sequence with an inducible promoter responsive to external control; delivering the expression sequence into the genome of the plant; and inactivating a second DNA molecule which codes for the product of BS92-7 from the native genome of the plant.

24. The method of claim 23 wherein the nucleotide sequences comprise the nucleotide sequences encoding the amino acids of SEQ ID Nos. 2, 4 or 8 or having the nucleotide sequence of any of SEQ. ID Nos. 1, 3, or 7 and those nucleotide sequences which hybridize to any of said sequences.
25. A method of reproducing a plant having heritable, externally controllable male sterility produced according to the method of Claim 23 further comprising planting seed of the plant to provide growing male sterile plants; inducing conversion of the growing plants to male fertile form under conditions which induce the promoter to express the first DNA molecule; and open-pollinating the growing plants in isolation to produce seed; and harvesting the seed.
26. A controllably male sterile plant produced according to the method of Claim 23.
- ~~24-27.~~ An expression vector comprising the DNA sequence of Claim 1.
- ~~25-28.~~ The expression vector of claim 27 further comprising an exogenous gene, wherein the exogenous gene is operably linked to the promoter.
- ~~26-29.~~ The expression vector of claim 27 wherein the promoter is selected from any one of CaMV35S, SGB6, BS92-7, MS45 or 5126.
- ~~27-30.~~ The expression vector of claim 27 wherein the product of the exogenous gene disrupts the function of male tissue.
- ~~28-31.~~ Plant cells comprising the vector of claim 27.
- ~~29-32.~~ A method of mediating male fertility in a plant comprising introducing into a plant the expression vector of claim 27 wherein the exogenous gene impacts male fertility of the plant and the promoter controls expression of the exogenous gene.
- ~~30-33.~~ The method of claim 32 wherein a regulatory element in conjunction with the promoter is inducible.
34. A nucleotide sequence as represented in ATCC deposit no. 98932.
35. An isolated nucleic acid sequence encoding the promoter region of the gene BS92-7.
36. An isolated nucleic acid sequence comprising SEQ ID NO. 5 or those nucleotide sequences which hybridize to SEQ ID NO. 5 under conditions of high stringency.
37. A male tissue-preferred regulatory region comprising nucleotide sequences essential for initiating transcription of the BS92-7 gene.

38. A male tissue-preferred regulatory region comprising Seq. ID. NO. 6.
- ~~36-39.~~ An isolated nucleic acid that is a male tissue specific regulatory element comprising the nucleotide sequence of SEQ ID No. 6 or those nucleotide sequences which hybridize to SEQ ID. NO. 6 under conditions of high stringency wherein the regulatory element is essential for initiating transcription of Seq ID Nos. 1, 3 or 7.
- ~~63-40.~~ The regulatory region of claim 37 comprising nucleotide sequences of about 130 contiguous base pairs upstream of the TATA box of the BS92-7 gene.
- ~~38-41.~~ A regulatory region comprising the sequences of -141 to -83 upstream of the TATA box of SEQ ID NO. 7 or those nucleotide sequences which hybridize to said sequences under conditions of high stringency
- ~~39-42.~~ The regulatory region of claim 41 comprising sequences -112 to -93 upstream of the TATA box of SEQ ID. No. 7 or those nucleotide sequences which hybridize to said sequences under conditions of high stringency.
- ~~40-43.~~ The regulatory region of claim 41 comprising sequences -161 to -152 upstream of the TATA box of SEQ. Id. No. 7 or those nucleotide sequences which hybridize to said sequences under conditions of high stringency
- ~~41-44.~~ The regulatory region of claim 41 comprising sequences -141 to -132 upstream of the TATA box of SEQ. Id. No. 7 or those nucleotide sequences which hybridize to said sequences under conditions of high stringency
- ~~42-45.~~ The regulatory region of claim 41 comprising sequences -92 to -83 upstream of the TATA box of SEQ. Id. No. 7 or those nucleotide sequences which hybridize to said sequences under conditions of high stringency
- ~~43-46.~~ An expression vector comprising a promoter that is operably linked with the male tissue specific regulatory element of claim 37.
- ~~44-47.~~ The expression vector of claim 46 further comprising an exogenous gene, wherein the exogenous gene is operably linked to the promoter.
- ~~45-48.~~ The expression vector of claim 46 wherein the promoter is selected from any one of CaMV35S, SGB6, BS92-7 MS45 or 5126.
- ~~46-49.~~ The expression vector of claim 46 wherein the product of the exogenous gene disrupts the function of male tissue.

~~47-50.~~ Plant cells comprising the vector of claim 46.

~~48-51.~~ A method of mediating male fertility in a plant comprising introducing into a plant the expression vector of claim 46 wherein the exogenous gene impacts male fertility of the plant and the regulatory element in conjunction with the promoter control expression of the exogenous gene.

~~49-52.~~ The method of claim 51 wherein the exogenous gene disrupts function of male tissue of the plant causing the plant to be male sterile.

~~50-53.~~ The method of claim 52 wherein a (?) regulatory element in conjunction with the promoter is inducible.

~~51-54.~~ The method of claim 51 wherein the plant is constitutively sterile when the promoter and regulatory element are not induced and is fertile when the promoter and regulatory element are induced.

~~52-55.~~ The method of claim 51 further comprising cross-fertilizing the male sterile plant with a second plant, the second plant comprising a second exogenous gene, the product of the second gene preventing disruption of the male tissue by the first heterologous gene, producing a male fertile hybrid plant.

~~53-56.~~ A method of producing hybrid seeds comprising: (a) producing a first parent plant comprising nucleotide sequences essential for initiating transcription of the BS92-7 gene operably linked with an exogenous gene impacting male fertility of the plant such that the plant is male sterile; (b) producing a second parent plant which is male fertile; (c) cross-fertilizing the first parent plant and the second parent plant to produce hybrid seeds.

~~54-57.~~ The method of claim 56 wherein the gene impacting male fertility is dominant and further comprising growing the hybrid seed to produce a third male sterile parent plant; producing a fourth parent plant comprising one or more genes controlling a desired gene trait and cross-fertilizing the third and fourth parent plants to produce second hybrid seed.

**ABSTRACT**

Nucleotide sequences mediating male fertility in plants are described, with DNA molecule and amino acid sequences set forth. Promoter sequences and their essential regions are also identified. The nucleotide sequences are useful in mediating male fertility in plants.